The Examiner rejected claims 1-9 under 35 USC § 112, second paragraph as indefinite. The Examiner asserts that the term "steric barrier lipid" is "uncertain as to meaning and scope." The Examiner has stated that the term "steric" has a certain dictionary meaning, but has not identified the dictionary on which he relies. Furthermore, he states that his dictionary defines "steric" as having to do with the "arrangement of atoms in space," and not as a material that provides a structural barrier that physically blocks. Applicants point out that a combination of the Examiner's definition with the word "barrier" which is part of the claims, results in a "barrier arising from the arrangement of atoms in space." This is plainly a structural barrier that leads to physical blockage. Furthermore, Applicants point out the attached definition from CancerWeb, in which the word "steric" is explained as:

> Steric hindrance, interference with or inhibition of a seemingly feasible reaction (usually synthetic) because the size of one or another reactant prevents approach to the required interatomic distance.

Again, this shows that the word's steric barrier fairly indicates that a steric barrier lipid is one that leads to a physically block due to the structure of the barrier lipid. Thus, Applicants believe that the meaning of the term "steric barrier lipid" is consistent with ordinary usage of the chemical terms. Nevertheless, they also attach several technical publications in which the concept of "steric barrier" lipids is discussed. For these reasons, Applicants submit that the rejection under 35 USC § 112, second paragraph should be withdrawn.

The Examiner maintained the rejection of claims 1-9 under 35 USC § 103 over Wheeler, pointing out that both the Wheeler application and this one refer to DOGS as an appropriate cationic lipid. Applicants thank the Examiner for pointing this out, since the inclusion of DOGS in this application was in error because DOGS is not an ionizable lipid that will work in the present invention. The present invention provides compositions in which external, non-encapsulated ODN are removed from the lipid particle by changing the pH to one near physiological pH, a pH at which the ionizable lipid has a substantially neutral charge.

DOGS is dioctadecylamidoglycylspermine and is sold under the trademark TRANSFECTAM by Promega. While this compound is ionizable in a broad sense, it is not in fact useful for the present invention because it retains substantial charge at physiological pH. For example, reflected in the attached copy of a reference by Barthel et al., page 555, 2mM DOGS

(referred to in the article as lipospermine) contains 6nM ammonium cationic charges at neutral pH. This positive charge is both essential for complexation of the DNA with the DOGS lipid, and facilitates the transfection process in which it is used in the Barthel article. In contrast, in the claimed invention it is desirable to eliminate external complexed DNA at physiological pH to provide a composition suitable for administration. Applicants would further note that this difference in the behavior of DOGS as a function of pH, makes it more like the permanent cationic lipids described in Wheeler, and does not suggest the ionizable lipids as now more clearly claimed, or specific ionizable lipids such as DODAP (claims 5 and 9).

In view of the fact that reference to DOGS in this application was incorrect, this reference has now been deleted, and the claims have been amended to clarify the nature of the ionizable lipid. The added definition of physiological pH added on Page 10 is taken from Parent Application Serial No. 09/078,954, (Page 12), which was incorporated by reference in this application, and therefore is not new matter. Since the error in mentioning DOGS appears to be the reason that the Examiner found the previous arguments unconvincing, Applicants believe that all of the claims of this application should be deemed allowable.

In view of the foregoing, reconsideration of the application and allowance of claims 1-9 are respectfully requested. Applicants have cancelled claims 10-22 without prejudice to facilitate prompt allowance of the application, and will file the promised terminal disclaimer upon receipt of an indication of allowability.

Respectfully Submitted,

Marina T Larson

Marina T. Larson, Ph.D.
Attorney/Agent for Applicant(s)
Reg. No. 32038

970 468 6600

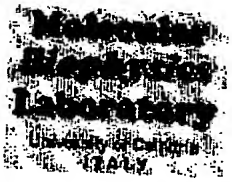
# steric

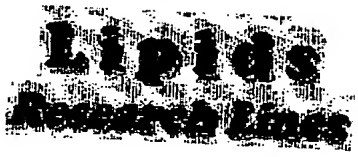
Pertaining to stereochemistry.

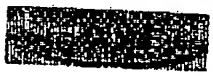
Steric hindrance, interference with or inhibition of a seemingly feasible reaction (usually synthetic) because the size of one or another reactant prevents approach to the required interatomic distance.

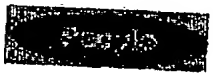
(05 Mar 2000)

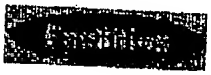
**Previous**: stereotyping, stereotypographer, stereotypography, stereotypy, sterhydraulic
**Next**: sterid, sterigma, sterigmatocystin

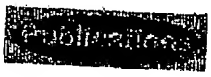


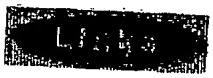



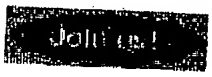



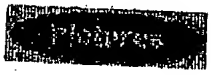


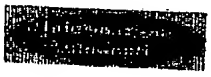



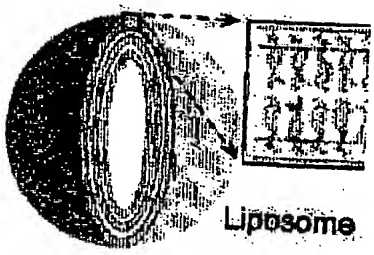



## MODEL MEMBRANES

The starting point of our researches has been the physical chacterization of lipid model membranes, obtained from the swelling in water of bilayer forming lipids, i.e., Phosphatidyl-cholines (**PCs**) and Phosphatidyl-ethanolamines (**PEs**).

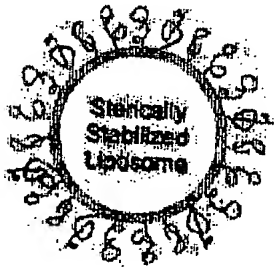



These lipids spontaneously aggregate in multilamellar structures (**MLVs**) (see top fi Extruding MLVs through polycarbonate membranes of defined pore size, i.e., 100 r possible to prepare Large Unilamellar Vesicles (**LUVs**). Alternatively, Small Unilam Vesicles (**SUVs**) could be obtained by ultrasonic rupture of MLVs with a sonicator.

With **conventional spin-label ESR spectroscopy**, **Saturation Transfer ESR (ST· spectroscopy** and **Optical spectroscopy** we have characterized all these model membranes alone and in interaction with: **Monovalent Cations, Anions, Chaotrop Poly(ethylene-glycol), Mitogens (Concanavalin A, Pytohemagglutinin), Anesth (Barbital, Tetracaine, Procaine), interdigitating agents (ethanol, glycerol).**

## STERICALLY STABILIZED LIPOSOMES

In the last years, our research interest has moved toward a novel class of lipid aggregates (**liposomes**) called either "Stealth" or "Sterically Stabilized Liposomes" (**SSLs**), obtained from the swelling in water of bilayer forming lipids and polymer-lipids, i.e., lipids having water-soluble polymers covalently attached on the polar head.

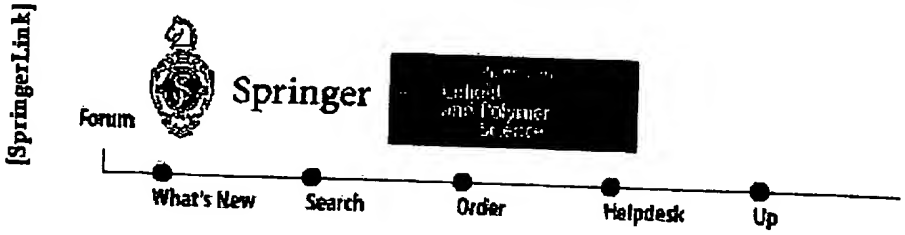



These polymer-grafted membranes, under particular conditions, act as very effectiv encapsulation and delivery systems. Their peculiar properties seem to arise from th steric barrier provided by the grafted polymers that stabilizes the lipid bilayer agains attacks of the diverse elements of the immune system *in vivo.*

Several parameters should be controlled in order to obtain sterically stabilized lipos able t incapsulate and to deliver water-soluble drugs: PEG-chain length, acyl chai length and degree of unsaturation of the PEG-phospholipid conjugate, and the acyl composition of th host bilayer matrix.

In our studies we are focusing on the phase- and the thermotropic behaviour of fully hydrated lipid/ polymer-lipid/water dispersions as a function of the polymer-lipid concentration in the system, the effects of the molecular weight of the hydrophilic p PEG (**PEG:350, PEG:2000, PEG:5000**) and the role played by the hydrophobic ch length of the phospholipids (**DMPC, DPPC, DSPC**) used on the polymorphism. Stu on the interaction of **Human Serum Halbumin (HSA)** with SSLs are also in progre

The research is mainly performed with **conventional spin-label ESR spectroscop** because this technique is especially suitable for studying lipid chain motions in fluid membranes, giving also information on lipid bilayers transition temperatures. **Satur transfer ESR (ST-ESR) spectroscopy** is used, in addition, because it is appropria studying the slow molecular motions of lipids in gel-phase membranes. **Optical spectroscopy** at fixed wavelength is used for evaluating dimensional variations of aggregates and lipid bilayers transition temperatures, too.

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**Abstract Volume 120 (2002) pp 35-40**
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# Steric stabilization of liposomes - a review

Mats Silvander

YKI, Institute for Surface Chemistry, Box 5607, 114 86, Stockholm, Sweden e-mail: mats.silvander@surfchem.kth.se Tel.: +46-8-7909803 Fax: +46-8-208998

**Abstract** The performance of two principally different kinds of intended stabilizers is reviewed. Especially any influence of the stabilizers on the liposome properties, such as structure, permeability and surface potential, is discussed. Poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymers have not been shown to function satisfactorily as stabilizers in combination with phospholipids. The incorporation of triblock copolymers of different segment length leads to structural breakdown of the liposome structure even at low concentrations. In addition, incorporation of the copolymers results in extensive leakage of encapsulated material. Neither have there been any reports of unambiguous proof of efficient steric repulsion due to the coating by copolymers. In contrast poly(ethylene glycol) lipids [PEG (2000)- phosphatidyl ethanolamine] efficiently provide a steric barrier to the liposomes. This is, however, only true if the surface concentration is kept below a rupture limit. An important additional effect is that the permeability of encapsulated hydrophilic cargo is reduced in the presence of PEG lipids. The most common PEG lipids contain a carbamate linkage that introduces a negative surface potential at the liposome surface. However, at medium ionic strength similar to physiological conditions the surface potential is small and does not contribute to any important extent to colloidal stabilization.

*Key words* Stealth · Colloidal stability · Poly(ethylene glycol) lipid · Pluronic · Vesicle

Article in PDF format

Online publication: May 17, 2002
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Next: Session *N7.2 Up: -MRS- Previous: Session N6.9

# Session *N7.1

**1:30 AM *N7.1**
SPONTANEOUS FORMATION OF LIPOSOMES WITH GRAFTED POLYMERS. I. Szleifer, Department of Chemistry, Purdue University, West Lafayette, IN.

Liposomes are closed spherical bilayer, formed by lipid molecules, that have potential use as drug delivery systems. Liposomes containing polymers grafted on their surfaces have been shown to have extended longevity in the blood stream. The increased longevity is believed to be due to the steric repulsion that the grafted polymer layer presents to proteins in blood and to cells. These liposomes are in general prepared by extrusion or sonication methods, i.e. they are formed by the insertion of energy on the system. The phase diagram of spontaneous forming liposomes in mixtures of lipid and lipid-poly ethylene oxide (PEO) molecules has been calculated using the single-chain mean-field theory. The theory predicts the minimal composition of lipid-PEO necessary to have stable liposomes as a function of the chain length of the polymer (PEO) chain. The predictions of the theory have been tested with experimental observations and there is very good agreement between the two. The driving forces for spontaneous liposome formation will be discussed. The spontaneously formed liposomes are predicted to have an asymmetric distribution of polymer chains between the inner and outer monolayers. The theory has been applied for a variety of polymer chain chemical architectures. In each case the range of spontaneous forming liposomes will be shown, and the use of different polymer structures for targeted drug delivery and their ability to provide the necessary steric barrier will be described. Thermodynamic stabilization of metastable liposomes by grafting polymers on the outer surface of the liposome will be shown for a variety of conditions. The ability to control the size of the spontaneous forming liposomes by the choice of the polymer molecule will be discussed in detail.

Next: Session *N7.2 Up: -MRS- Previous: Session N6.9
*System Administrator*
*11/13/1997*

*J. Am. Chem. Soc.* 1999, *121*, 3240−3241

# Magnetically Oriented Phosph lipid Bilayers for Spin Label EPR Studies

Seth M. Garber,† Gary A. Lorigan,‡ and Kathleen P. Howard*,†

*Department of Chemistry, Swarthmore College Swarthmore, Pennsylvania 19081*
*Department of Chemistry and Biochemistry Miami University, Oxford, Ohio, 45056*

*Received December 21, 1998*
*Revised Manuscript Received February 24, 1999*

This paper reports the development of a model membrane system that spontaneously orients in the applied magnetic field of an EPR spectrometer. There has been a great deal of excitement recently over the use of magnetically oriented phospholipid bilayers (bicelles) for both solution and solid-state NMR spectroscopy.[1−4] Here we report nitroxide spin label data from a novel bicelle system which orients in the lower magnetic field of a conventional EPR spectrometer (<1 T for an X-band EPR spectrometer as opposed to ~11.8 T for a 500 MHz NMR spectrometer). The system offers the opportunity to carry out EPR studies using a well-oriented highly hydrated model membrane system whose preparation is amenable to the reconstitution of labile membrane components such as integral membrane proteins. Furthermore, the magnetically orienting system reported here presents new opportunities to carry out both NMR and EPR studies on the same sample preparation providing complementary dynamic information in two different time scale regimes.

EPR studies of membrane systems utilizing nitroxide spin probes have greatly improved the view of the dynamic behavior of membrane components at a molecular level by providing information about the types and rates of motion, as well as the degree of organization of lipids.[5,6] Nitroxide spin labels are sensitive monitors of molecular organization and can provide detailed information for small quantities of biological systems. Nitroxide EPR studies have provided important insights into a wide range of membrane phenomena including information about lipid phase transitions[7] and information about how proteins interact with membrane lipids.[8]

It has long been known that the use of well-oriented multibilayers of lipids can greatly increase spectral resolution of EPR membrane studies as well as provide important data by allowing the collection of spectra obtained as a function of the angle between the magnetic field and the bilayers.[7,9,10] However, many of the existing alignment methods suffer from significant drawbacks such as labor intensive preparation, instability at high levels of hydration, and the requirement of procedures involving either organic solvents or elevated temperatures that can destroy labile membrane components such as proteins. Some procedures have been devised whose methods are more amenable to the study of membrane proteins. These methods include one study where whole cells were aligned in an external magnet (but not the magnetic field of the EPR spectrometer as reported here), followed by freezing of the sample before transferring the sample to an EPR spectrometer.[11] However, this method required freezing the samples and resulted only in partially aligned samples. Most recently Freed and co-workers reported the use of isopotential spin-dry ultracentrifugation to produce well-aligned films of both bacteriorhodopsin- and gramicidin-containing membranes.[12] This method enabled the collection of extensive oriented experimental data that, combined with quantitative theoretical analyses, provided a detailed description of protein/lipid interactions for membrane preparations containing 20 wt % water.

We think the method reported here offers significant advantages over previous alignment methods in terms of ease of preparation, stability at high degrees of hydration, and suitability for reconstitution of membrane proteins. The magnetically aligned samples described here are related to the systems initially introduced by Prestegard and co-workers[13] and subsequently adapted by Vold and co-workers[2] to align the bilayer normals of lipid bilayers so that they are parallel with the direction of the applied magnetic field. The oriented system we present here is composed of a mixture of a bilayer-forming phospholipid (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, DMPC) and a short chain phospholipid (1,2 dicaproyl-*sn*-glycero-3 phosphocholine, DHPC) that breaks up the extended bilayers into what has been described as disc-shaped micelles (bilayered micelles, or bicelles) that are highly hydrated (in this case 75% aqueous). To characterize this system for EPR spectroscopy, we have utilized a steroid derivative nitroxide spin probe called 3$\beta$-DOXYL-5$\alpha$-cholestane (cholestane). This rigid and elongated probe orients with its long axis parallel to the lipid acyl chains and reports on the general order of the lipids. The DMPC bilayers were enriched with 10% molar cholesterol to increase the local order in the membrane, restricting the movement of the spin probe and highlighting the effects of macroscopic bilayer orientation on the measured EPR spectra. The data reported here are from samples doped with $Yb^{3+}$ ions (10% molar to DMPC).[14] This system was used because the large magnetic susceptibility induced on the lipid bilayers by the $Yb^{3+}$ ions enabled it to be one of the easiest bicelle systems to orient in the magnetic field of an X-band EPR spectrometer.[15]

Crucial to the success of the experiment and stability of the sample was the addition of a small amount (1% molar to DMPC) of phospholipid that has a soluble poly(ethylene glycol) polymer tail attached to its headgroup, 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)2000] (PEG2000-

* To whom correspondence should be addressed. E-mail: khoward1@swarthmore.edu.
† Swarthmore College.
‡ Miami University.

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(15) Oriented bicelles were prepared as follows. DMPC, cholesterol, and PEG2000-PE were co-solubilized in chloroform, rotovapped, and then placed under high vacuum for at least 8 h. In a separate flask, DHPC and cholestane spin label were also co-solubilized in chloroform, rotovapped, and then placed under high vacuum for at least 8 h. Half of the 100 mM HEPES buffer needed for the final solution was added to the flask containing DHPC/cholestane and the other half added to the flask containing DMPC, cholesterol, and PEG2000-PE. Both flasks then went through several cycles of vortexing and warming until all of the dried material was released from the sides of the flask. DHPC/cholestane solution was then added to the flask containing DMPC, cholesterol, and PEG2000-PE and vortexed until the sample was homogeneous. Finally, the solution went through two freeze/thaw cycles using liquid nitrogen. Ytterbium chloride hexahydrate was added as an aqueous solution. Samples were then transferred to a quartz flat cell for EPR spectroscopy. DMPC, DHPC, and PEG2000-PE were purchased from Avanti Polar lipids. All other materials were purchased from Sigma/Aldrich.

10.1021/ja984371f CCC: $18.00 
Published on Web 03/19/1999

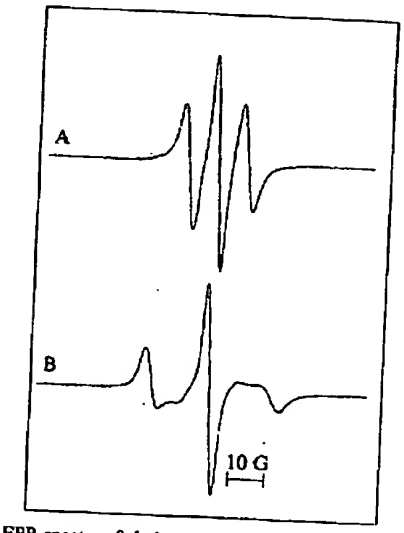


**Figure 1.** EPR spectra of cholestane incorporated into (A) macroscopically oriented bilayers composed of DMPC/DHPC/cholesterol/$YbCl_3$/PEG2000-PE/cholestane in the molar ratios 3.5/1.0/0.35/0.35/0.035/0.0056 in 100 mM HEPES buffer, pH 7.0. (B) multilamellar liposomes at 25% w/v with the same composition of (A) except DHPC was not included. Both spectra collected at 313 K on a X-band Bruker ER200D spectrometer (standard $TE_{102}$ cavity) equipped with a variable temperature setup. Spectra were collected with a sweep width of 100 G, center field was 3355 G, 100 kHz field modulation frequency, scan time ~4 min and a microwave frequency of 9.5 GHz under nonsaturating conditions (signal intensity would still increase with increasing microwave power). Because the two spectra were calculated from two samples with slightly different volumes, an accurate comparison of spectral intensities is difficult. Peaks heights were thus normalized to give the same peak height of the center peak in both spectra. (However, our experience is that oriented samples gave higher relative spectral intensities than unoriented samples for the same amount of spin label.) The samples were equilibrated in the EPR magnet at 7400 G for 2 h prior to collection of spectra.[27,28]

PE). We added PEG-derivatized lipid to follow the lead from the literature on drug delivery liposomes which has demonstrated that PEG-PE provides a strong steric barrier that prevents close contact of membrane surfaces of liposomes and cells and prolongs liposome circulation time.[16] Before we began adding PEG2000-PE to our spin-labeled bicelle samples, our lanthanide-containing samples showed evidence of precipitation within a few hours of preparation and either oriented poorly or not at all. Once we started adding PEG2000-PE to the bicelles, the samples showed high degrees of orientation and were stable over several days. Presumably PEG2000-PE prevents neighboring bicelle surfaces from approaching each other and fusing together to produce insoluble aggregates. We have also found that PEG2000-PE can be used for a range of different bicelle samples used for NMR studies and will offer advantages in other membrane bicelle preparations.[17]

Figure 1 clearly demonstrates the effects of macroscopic orientation on the EPR spectra of cholestane spin label in cholesterol-containing DMPC bilayers. The EPR spectra of nitroxide spin probes consist of a triplet due to the hyperfine interaction between the nuclear spin of the nitrogen ($^{14}N$) nucleus and that of the unpaired electron. At a microwave frequency of 9.5 GHz (~0.3 T magnetic field), the anisotropic contribution to the spectra is dominated by this hyperfine interaction. The standard system of coordinates for the N−O paramagnetic moiety is with the $x$-axis along the N−O bond and the $z$-axis along the nitrogen $2p\pi$ orbital containing the unpaired electron density.[18] Typical principal values for the hyperfine tensor of the cholestane spin label in lipid bilayers are $A_{zz} = 33.8$ G, $A_{xx} = A_{yy} = 5.0$ G.[7,19] For cholestane, the nitroxide $y$-axis is approximately parallel to the long axis of the probe. Previous work has demonstrated that cholestane aligns with it long axis parallel to the long axis of the lipids and undergoes rapid rotation about this axis.[20] The reduction of the hyperfine splitting in Figure 1A with respect to the unoriented sample in Figure 1B is clearly indicative of macroscopic orientation of the membrane bilayers with their normals (and hence $y$-axis of associated cholestane spin labels) parallel to the applied magnetic field. The "apparent" splitting (measured splitting between low field and middle field spectral lines) in the oriented spectrum in Figure 1A is 8.7 G, which is in close agreement with values measured in previous published spectra with cholestane spin label incorporated into oriented ~10 mol % cholesterol containing phospholipid bilayers on glass plates.[20,21]

The magnetic ordering methodology described here provides a promising means of further extending high-resolution oriented EPR methods to a wider range of protein-containing membrane systems. As already demonstrated in several NMR studies, the lipid bilayers in magnetically oriented bicelles are in the liquid crystalline phase and have the capacity to incorporate membrane proteins.[22−24] Furthermore, this magnetically orientable system can be used for both NMR and EPR studies, and several published reports have already explicitly called for studies on phospholipid−protein interactions that combine well-resolved $^2H$ NMR results with EPR spin label studies.[6,12]

**Acknowledgment.** This research was supported by Grant GM57627-1 to K.P.H. from the National Institutes of Health.

JA984371F

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(27) At 3400 G with 10% $Yb^{3+}$, the sample was partially oriented after 30 min. However, after the sample equilibrated in the magnet for 2 h with the field turned up to 7400 G, the spectral features characteristic of powder patterns disappeared. At higher concentrations of $Yb^{3+}$, orientation occurred much faster and at a lower magnetic field (3400 G); however, spectral broadening occurred presumably due to the interaction of $Yb^{3+}$ with the nitroxide moiety of the cholestane spin labels (which has been shown to be at the level of the carbonyl groups of the lipids composing the bilayers[12]). Other spin labels that are buried deeper in the lipid bilayer should not be broadened to the same degree.
(28) Since the degree of orientation increases with the square of the magnetic field,[13] the use of high-field EPR spectroscopy holds promise. In addition to ordering effects, at higher magnetic fields the nitroxide Zeeman interaction exceeds that of the nitrogen hyperfine interaction, and since the $g$-tensor of nitroxide radicals is nonaxial, this opens the possibility of being able to study features of lateral and transverse ordering directly.[25, 26]

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# LABORATORY METHODS

# Gene Transfer Optimization with Lipospermine-Coated DNA

FABRICE BARTHEL,[1] JEAN-SERGE REMY,[2] JEAN-PHILIPPE LOEFFLER,[1] and JEAN-PAUL BEHR[2]

## ABSTRACT

**Designed synthetic DNA carriers represent an attractive alternative to the widely used calcium phosphate gene transfer technique. In this context, we developed a class of nucleic acid binding lipids, the lipopolyamines, which spontaneously condense DNA on a cationic lipid layer. The resulting nucleolipidic particles transfect most animal cells efficiently. However, compaction depends on many experimental factors, some of which have been varied here to give optimal transfection efficiency. When plasmid condensation by the lipospermine is performed in the absence of competing polyions or serum proteins, or when the gene of interest is diluted into carrier DNA, transfection efficiency is increased by 2–3 orders of magnitude. With these improvements, chloramphenicol acetyl transferase activity resulting from transfection of as little as 25 ng could easily be detected by a nonradioactive ELISA test.**

## INTRODUCTION

GENE TRANSFER is among the most powerful techniques used in cell biology, allowing entry into various research fields (gene/protein function and regulation), as well as into biotechnology (livestock and crop improvement) and health, as a prerequisite to gene therapy. Many transfection techniques have been developed with variable success (for review, see Keown *et al.*, 1990), of which the straightforward calcium phosphate coprecipitation, electroporation, and recombinant viruses remain the most utilized. Comparatively, the rational design of *synthetic* chemical vectors is poorly documented (Felgner *et al.*, 1987; Wu and Wu, 1988; Behr *et al.*, 1989; Pimnaduwage *et al.*, 1989; Leventis and Silvius, 1990; Wagner *et al.*, 1990; Gao and Huang, 1991; Kabanov *et al.*, 1991; Rose *et al.*, 1991; Zhou *et al.*, 1991). In this context, we have described the synthesis and complexing properties of lipopolyamines toward DNA (Behr, 1986), leading to their use as versatile gene transfer agents (Behr *et al.*, 1989; Loeffler *et al.*, 1990; Demeneix *et al.*, 1991).

Here we present results of a careful optimization of the experimental factors expected to influence transfection efficiency. These improvements make lipospermine-coated DNA an even more attractive straightforward alternative to classical transfection techniques.

## MATERIALS AND METHODS

### *Cell culture*

*Cell Lines:* AtT20 and 3T3 cell lines were cultured in 80-cm² flasks (NUNCLON) in Dulbecco's modified Eagle medium (DMEM, GIBCO, Cergy-Pontoise, France) supplemented with 10% calf serum (GIBCO), 100 UG/ml streptomycin (GIBCO), 100 IU/ml penicillin (GIBCO), 0.1 mg/ml kanamycin (Intermed, Noisy-le-Grand, France), 0.286 gram/liter glutamine (Lancaster Synthesis, Morecambe, UK), and 2 grams/liter glucose (Janssen, Noisy-le-Grand, France) for 3T3 cells (4.6 grams/liter for AtT20) at 37°C in a humidified atmosphere containing 5% $CO_2$. At confluence, the cells were trypsinized and seeded at approximately 25–30% confluence in six-well dishes (Falcon) in 2 ml of culture medium.

*Primary Cultures of Cerebellar Granule Cells:* Granule cells were obtained from 6-day-old rats as described previously (Loeffler *et al.*, 1990). Tissue was kept in imidazole/NaOH-buffered DMEM pH 7.4. After mechanical dispersal, cells were resuspended in DMEM containing a high concentration of $K^+$ (30 m*M* final concentration) and supplemented with 50 μg/ml gentamicin (Sigma), $5 \times 10^{-7}$ *M* insulin (SIGMA), and 10% heat-inactivated horse serum (GIBCO). Cells were plated at a density of $5 \times 10^6$ cells/

[1]Institut de Physiologie, URA 1446 du CNRS, F-67000 Strasbourg, France.
[2]Laboratoire de Chimie génétique, URA 1386 du CNRS, Faculté de Pharmacie, F-67401 Strasbourg, France.

dish on polyornithine-coated dishes (6 3.5 cm) and grown at 37°C in a humidified atmosphere (5% $CO_2$). This procedure results in an almost pure culture (>95%) of differentiated neurons as verified by GFAP and neuron-specific enolase immunostaining after 3 days in culture.

### *Plasmids and transfecting agent*

The bacterial chloramphenicol acetyl transferase (CAT) reporter gene was used throughout this study. This enzyme, which reflects transcriptional activity of a given promoter, is easily detectable by a sensitive assay and the results can be quantified.

Plasmids were propagated and purified by standard techniques (Maniatis *et al.*, 1982). In this study, we investigated basal expression of various CAT constructs driven by viral or heterologous promoter sequences: pSV-CAT, simian virus 40 early promoter; RSV-CAT, Rous sarcoma virus; CMV-CAT, cytomegalovirus; 4xTRE-tk/CAT, four collagenase gene TPA-responsive elements (TGACTCA) inserted 5' to the herpes simples virus thymidine kinase (tk) gene minimal promoter.

The lipospermine DOGS (Fig. 1) was synthesized according to Behr *et al.* (1989); this compound is also commercially available from Promega, USA (Transfectam, registered TM of IBF-Sepracor, France).

### *Transfection procedures* (see also Discussion section for a more general protocol)

*2XDMEMp Protocol:* Two micrograms of plasmid and 4 μl of a 2 m*M* Transfectam solution (in ethanol) were each added to 250 μl 2× DMEMp (in which the divalent cation concentration was depressed by a freeze/thaw cycle and filtration) and vortexed. After ~10 min, the two solutions were mixed and vortexed. After another 10 min, 500 μl of distilled water was added and the solution was vortexed. This mixture (1 ml) was poured over the cells (usually washed twice with DMEM if cultured in presence of serum). The transfection medium was left for a variable time and cells were returned to normal culture medium.

*NaCl Protocol:* Two micrograms of plasmid and 4 μl of a 2 m*M* Transfectam solution (in ethanol) were diluted separately into 2 × 50 μl of 150 m*M* NaCl and vortexed. After ~10 min, the two solutions were mixed and vortexed. After another 10 min, 900 μl of culture medium (with or without serum) was added and the solution was homogenized. This transfection medium was added to the cells 10 min later.

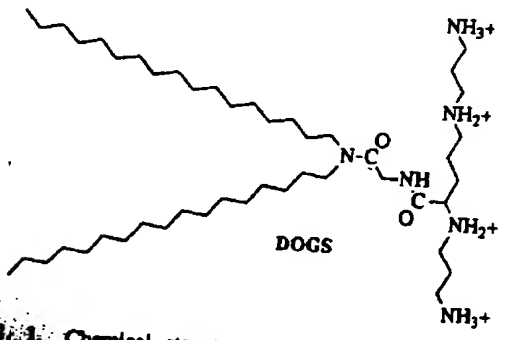


FIG. 1. Chemical structure of the lipospermine used throughout this work.

### *Enzymatic CAT assay*

CAT activity was determined by the method of Gorman *et al.* (1982). Cells were suspended in 100 μl of 200 m*M* Tris-HCl pH 7.4. After several freeze/thaw cycles, the extract was heated (65°C) for 10 min, centrifuged (10,000 rpm, 5 min) and 80 μl of the supernatant was added to 40 μl of Tris-HCl containing [$^{14}$C]chloramphenicol (0.05 μCi per sample; sp. act., 47 mCi/mmole). After 5 min at 37°C, the reaction was initiated by adding 40 μl of 4 m*M* acetyl-coenzyme A. The mixture was kept at 37°C for 2 hr and then extracted with 0.5 ml of ethyl acetate. After separation by silicagel thin-layer chromatography (chloroform/methanol, 95:5), the acetylated and unreacted forms of chloramphenicol were located by autoradiography, cut out, and counted. With constant initial amount of substrate, CAT activity was quantified as percent of chloramphenicol converted to acetylated forms.

### *CAT ELISA test*

Immunological quantification of CAT was performed with a commercial ELISA test (Boehringer, France) according to the manufacturer's instructions. Cells were thawed and lysed with 200 μl of lysis buffer. After centrifugation, 80 μl (more or less if outside the calibration curve) was used for CAT-ELISA test as described by Boehringer. Briefly, the volume of cell extract was completed to 200 μl with sample buffer, added to rehydated precoated wells, and incubated for 1 hr at 37°C. The cell extracts were discarded and the wells were washed three times with washing buffer. Two-hundred microliters of anti-CAT-DIG (2 μg/ml) was added into each well and the plate was incubated for 1 hr at 37°C. The anti-CAT-DIG was discarded and the wells were washed again as described above. Then, 200 μl of anti-DIG-POD (150 mU/ml) was added into each well and the plate was incubted for 1 hr at 37°C. After another washing cycle, 200 μl of POD substrate was added and the plate was incubated for 1 hr at room temperature. The $OD_{405\,nm}$ of the samples was converted to picograms of CAT per well with a calibration curve of the native enzyme (typically linear from 20 pg up to 500 pg per cell extract).

## RESULTS

Lipopolyamines condense plasmid DNA and coat it with a cationic layer following simple mixing. The resulting multimolecular particles probably enter the cell *via* spontaneous endocytosis. Apart from the lipid structure (depicted in Fig. 1), the level of transfection will depend on measurable parameters like the amount of DNA, the ratio of lipid

ammonium to DNA phosphate charges, the incubation time (some of which were already explored; Behr *et al.*, 1989), but also on more diffuse environmental conditions, for example those governing the lipospermine/DNA compaction step or the cationic particle/cell-surface interaction.

### *General transfection conditions*

Most transfection experiments were performed on murine AtT20 cells (derived from a pituitary tumor) and 3T3 fibroblasts, with the TRE-tK/CAT plasmid (containing 4× the TPA-responsive element upstream of the HSV thymidine kinase promoter). These enhancer elements confer high basal expression in most cells and thus allow rapid and convenient screening of transfection conditions. The general meaning of the conclusions reached by optimizing compaction and transfection conditions on these plasmid/cells combinations was checked with other reporter constructs, where the CAT gene is under the control of constitutive viral promoter sequences (SV40, RSV, CMV; see Materials and Methods), as well as with other cells (HepG2 hepatocytes, L929 fibroblasts) (data not shown). Along the same lines, the mean transfection efficiencies and standard deviations given in the figures are generally the result of at least three experiments of which at least half are truly independent.

Although we focus here on a global measure of *transient* expression in cell lines requiring in principle no cell division, transfection efficiency may depend indirectly on cell division (and on the cell surface-medium contact) since actively growing cells can be expected to have surface properties different from resting cells, and some stages of the cell cycle are probably more "competent" for gene transfer than others (Hanahan, 1985). Indeed, preliminary experiments with rapidly growing L929 fibroblasts showed that confluent (hence growth-arrested) TRE/tK-CAT-transfected cells hardly express CAT, a fact that may be interpreted in the light of the above hypothesis, or alternatively reflect low nuclear API binding activity. To avoid such cell cycle-dependent interferences, all experiments were performed under standard conditions, at 30–50% confluence 24 hr post-plating; in addition, the cells were rinsed prior to transfection to avoid any unpredictable interference with serum or cell-released compounds.

### *Ratio of lipid to plasmid*

Various quantities of Transfectam were added to a given amount of DNA to assess the influence of the particles mean electric charge on transfection. The results are plotted in Fig. 2 as the CAT activity *versus* the excess of cationic charges calculated on the following basis: 1 μg of DNA contains 3.1 nanomoles phosphate anionic charges (assuming a mean molecular weight of 325 for a nucleotide sodium salt); 1 μl of a 2 m*M* (2.5 mg/ml Transfectam tetratrifluoroacetate of $M_w$ = 1,263) lipospermine solution contains 6 n*M* ammonium cationic charges at neutral pH. Cell lines and primary cultures show the same trends: the exogenous enzymatic activity is undetectable until half-

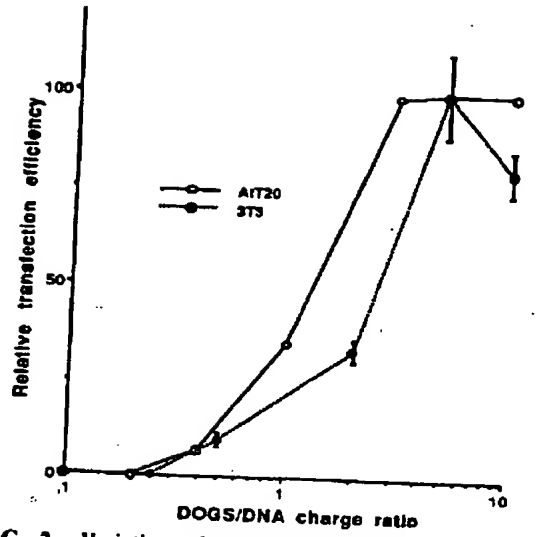


**FIG. 2.** Variation of transfection efficiency with the mean charge of the DNA–lipospermine complexes. Cells were transfected with 2 μg of DNA and increasing amounts of Transfectam. The NaCl transfection protocol was used for 3T3 cells (see Materials and Methods); the AtT20 transfection protocol is described elsewhere (Behr *et al.*, 1989). After 10 hr of incubation, the medium was replaced by DMEM (AtT20) or 10% serum-supplemented DMEM (3T3) for 24 h. Results are expressed as relative transfection efficiency for each cell line and means ± SE are given ($n$ = 3) for 3T3 cells.

neutralization and increases suddenly around neutrality. Transfection becomes really efficient only when the lipopolyamine/plasmid complex bears a strong positive charge; when this condition is fulfilled, transfection is almost independent of the amount of lipopolyamine, although a slight decrease is observed at the highest ratios. This latter effect may be attributed to the onset of cytotoxicity at a concentation which varies for each cell type.

### *Dose dependency*

To avoid misinterpretation arising from a variable net charge (see above), the DNA dose dependency was not checked by adding increasing amounts of DNA to a given amount of lipopolyamine, but rather by plotting transfection efficiency against the amount of DNA/Transfectam complex, at a charge ratio where it is actively taken up. Figure 3 shows a transfection threshold around 1 μg of added DNA. Interestingly, this phenomenon disappears in favor of a more classic dose-response figure when the plasmid of interest (CAT reporter gene) is diluted in a constant amount of carrier plasmid DNA (pBR322). The net result of dilution is a much more sensitive technique, since transcription resulting from transfection of as little as 25 ng reporter DNA could be detected (Fig. 3, inset).

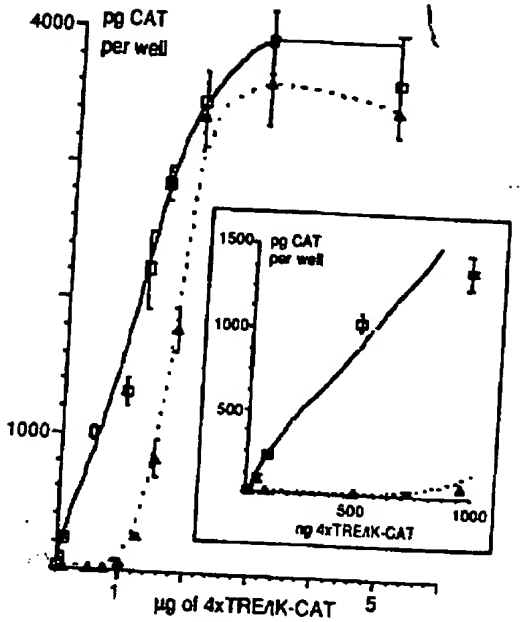


FIG. 3. Plot of the transfection level *versus* the amount of DNA–Transfectam complexes at 6× cationic charge excess. The NaCl transfection protocol was used to transfect 3T3 cells. The transfection medium contained 10 ng to 5 µg 4× TRE-tK/CAT reporter gene and 3 µl/µg DNA of a 2 m*M* ethanolic Transfectam solution (triangles). Alternatively, 10 ng to 5 µg of 4× TRE-tK/CAT were complemented to 5 µg of total DNA with pBR322 and 15 µl of Transfectam solution was added (rectangles). After 8 hr, the medium was replaced by DMEM complemented with 10% FCS, for 24 hr. Results were quantified by CAT ELISA and expressed as picograms of CAT per well for each condition. Means ± SE are given ($n$ = 3).

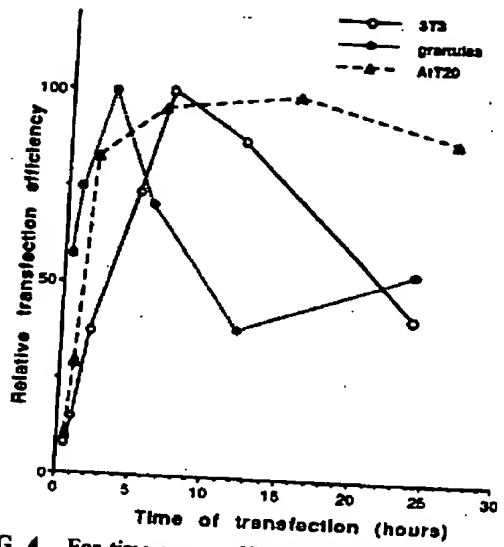


FIG. 4. For time-course efficiency, cells were incubated with 2 µg of 4× TRE-tK/CAT–Transfectam complexes having a four-fold (AtT20 and granule cells) or six-fold (3T3 cells) charge excess. The complexes were obtained with the 2× DMEMp (AtT20, granule cells) or NaCl (3T3) transfection protocols. After the indicated time period, the medium was changed for DMEM (AtT20 and granule cells) or DMEM complemented with 10% FCS (eTe), and CAT activity was determined after 48 hr. Results are expressed as relative transfection efficiency for each cell type ($n$ = 3). Means (± 15%) are given.

### *Incubation time*

The efficiency of transfection depends on the incubation time, *i.e.*, the time after which the transfection mixture was removed from the cells and replaced by serum-supplemented culture medium (Fig. 4). The efficiency reaches a plateau and possibly decreases for longer times. This latter effect may reflect cytotoxicity or may be a consequence of prolonged culture in the absence of serum. The optimal transfection time varies with the cell type, ranging from 3 hr (primary cerebellum) to ~7 hr (3T3 and AtT20).

### *Kinetics and environment of DNA condensation by the lipospermine*

Plasmid compaction by Transfectam was assayed by [illegible] dilution of both partners before mixing and final [illegible] the medium to make it isotonic with the cells. [illegible] diffusion kinetic experiments had shown [illegible] occurs within a few minutes (data not shown). Therefore, to assess empirically the influence of condensation time on transfection, the following series of experiments was planned: constant aliquots of DNA and DOGS from stock solutions were diluted separated into 250 µl of 2× DMEMp; immediately or after ~10 min, the solutions were vortexed together to allow for DNA/Transfectam complexation to occur. After 0–10 min incubation, the mixture was diluted with 500 µl of water, and poured over the cells after another 0–10 min incubation. The resulting CAT activities are plotted in Fig. 5 relative to that of the longest experiment (3× 10 min), and show no drastic influence of these incubation times provided not all of them are short (3× 0 min).

For convenience, transfection experiments requiring mixture of a plasmid with another compound (whether calcium phosphate, Polybrene, DEAE-dextran, cationic lipids, or even asbestos fibers) are generally performed in a cell culture medium containing many potentially interfering compounds; the situation is even worse if the culture medium is serum supplemented. Lipospermine-mediated transfection was originally described in Dulbecco's modified Eagle medium (DMEM). Taking this as a reference, we tried to improve the transfection efficiency by changing the ionic conditions of the DNA/Transfectam condensation step (Fig. 6). Water and twofold increase in ionic strength (2× DMEM) gave almost an order of magnitude increase in efficiency. This latter concentrated medium was

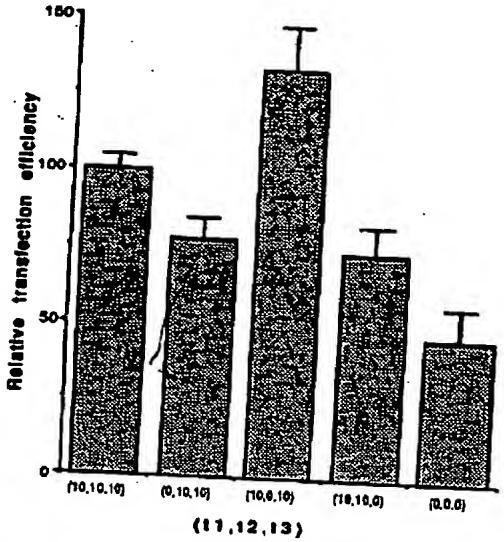


FIG. 5. Incidence of the kinetics of complex formation on the transfection efficiency. A total of 2 $\mu$g DNA and 4 $\mu$l of an ethanolic 2 m*M* Transfectam solution were diluted separately in 250 $\mu$l of 2× DMEMp medium (see Materials and Methods) and vortexed. After 0 or 10 min (t1), the solutions were mixed. After a further 0 or 10 min (t2), 500 $\mu$l of distilled water was added to give a final 1× DMEM transfection medium that was poured onto the cells after 0 or 10 min (t3). After 12 hr of incubation, the medium was replaced by DMEM for 24 hr. Results are expressed as transfection efficiency relative to the longest experiment (t1 = t2 = t3 = 10 min). For each condition, the mean ± SE is given.

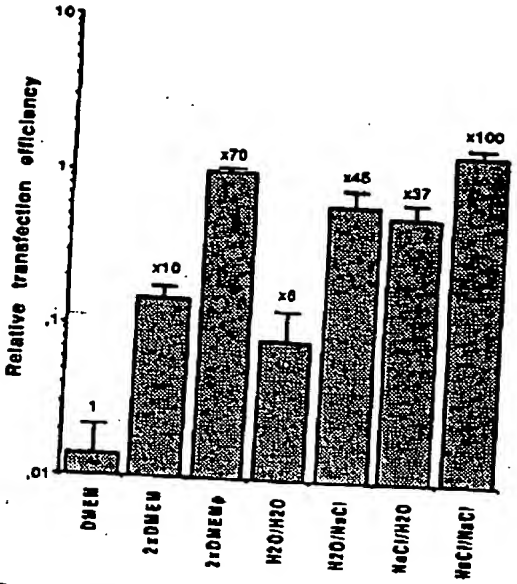


FIG. 6. Incidence of the complex formation medium on the transfection level. The various transfection media containing 4 $\mu$l of a 2 m*M* solution of Transfectam and 2 $\mu$g 4× TRE-tK/CAT reporter gene were prepared as follows (dilutions and complex formation steps were of 10 min): DMEM: DNA and Transfectam were each diluted in 500 $\mu$l DMEM, then mixed, and poured onto the cells. 2×DMEM: freshly prepared 2× DMEM was used. DNA and Transfectam were each added to 250 $\mu$l of this medium. Solutions were mixed; 500 $\mu$l of distilled water was added. 2×DMEMp: was obtained from 2× DMEM after a freeze/thaw cycle followed by filtration; it was used as 2× DMEM. $H_2O/H_2O$: DNA and Transfectam were each added to 50 $\mu$l of distilled water; the diluted solutions were mixed, and 900 $\mu$l of DMEM was added. $H_2O$/NaCl: DNA was added to 50 $\mu$l of distilled water and Transfectam to 50 $\mu$l of 300 m*M* NaCl; the solutions were used as above. NaCl/$H_2O$: DNA was added to 50 $\mu$l of 300 m*M* NaCl and Transfectam to 50 $\mu$l of distilled water, and used as above. NaCl/NaCl: DNA and Transfectam were each added to 50 $\mu$l of 150 m*M* NaCl solution, and used as above. For each condition, mean ± SE is given ($n$ = 6).

incidently frozen and left a small precipitate upon coming back to room temperature; the supernatant showed to be a still more favorable compaction medium: another order of magnitude was gained (2× DMEM vs 2× DMEMp). Among all ions present in DMEM, we suspected calcium hydrogenphosphate to be the ionic combination likely to precipitate out at neutral pH ([$CaCl_2$] = 4 m*M*, [$NaH_2PO_4$] = 2 m*M* in 2× DMEM. solubility product [Ca]·[HPO4] = 1.7 m*M* at R.T.). Indeed, compaction in a simple isotonic medium devoid of alkaline-earth cations (NaCl, Fig. 6) gave the same enhanced transfection level; addition of $CaCl_2$ (2 m*M*), and to some extent $NaH_2PO_4$ (2 m*M*), to NaCl or DMEMp severely decreased this gain (data not shown).

### *Influence of serum on transfection*

Most cells in culture require serum for optimal growth and the efficiency of various transfection methods has been shown to be affected by serum. Preliminary experiments where DNA coating and cell transfection were done in 10% serum-supplemented DMEM showed a severe loss in efficiency as compared to DMEM. To [illegible] interference on complex formation from [illegible]

face binding, and to avoid misinterpretation due to growth factor-induced plasmid transcription, the following experiments were compared: DNA/Transfectam mixing in the presence or absence of 10% fetal calf serum in 2× DMEMp or in 150 m*M* NaCl), 8 hr cell incubation with or without serum, followed by 24 hr [illegible] serum-supplemented DMEM. When serum [illegible] during complexation, the transfection efficiency [illegible] when present [illegible]

## DISCUSSION

Lipospermine-mediated transfection is based on nucleic acid condensation by the polycationic lipid (Behr, 1986). Indeed, the spermine head group binds strongly (Braunlin *et al.*, 1982) but without sequence selectivity to the DNA minor groove (Schmid and Behr, 1991), and this interaction becomes cooperative and irreversible due to the lipid–lipid neighborhood. Thus, after mixing of a plasmid solution with excess lipospermine (structure in Fig. 1), small cationic particles form spontaneously (as depicted schematically in Fig. 7; notice that although lipid mediated, this is not a liposome-based technique and requires no encapsulation step). These compact nucleolipidic particles are attracted to the cell surface by electrostatic interaction and eventually enter the cell by zipper-like endocytosis (Griffin *et al.*, 1975; Haywood, 1975; Behr, 1993). The mechanisms of subsequent uncoating, nuclear membrane crossing, and foreign gene expression remain obscure as for most other transfection techniques. However, this method is straightforward (simple mixing of components), general with respect to the cell line, since driven by a non-specific interaction, and of low toxicity due to a potentially biodegradable lipid vector possessing two peptide bonds.

### *Efficient transfection requires compacted "cationic plasmids"*

Many transfection techniques rest with the interaction of polyanionic DNA either with a cationic polymer (DEAE-dextran, Polybrene, polylysine, *etc.*), with an aggregated cationic amphiphile, with the cationic surface of an inorganic particle (asbestos fiber), or with coprecipitation of an inorganic salt ($Ca^{2+}$, $Sr^{2+}$, $Ba^{2+}$, $Mn^{2+}$). In any case, the polyionic interaction leads to an almost irreversible aggregation into particles of variable size able to transfer the plasmid into cells, in contrast to the reversible condensation induced by polyamines or $Co(NH_3)_6^{3+}$ (Arscott and Bloomfield, 1990), which are unable to promote transfection (Remy, unpublished). This background suggests some common features between cation-mediated gene transfer methods, their individual efficiencies relying possibly on variable particle size, protecting coat, cytotoxicity, *etc.* We speculate that all these methods provide a polycationic surface able to condense DNA and to make an ionic link between the polyanionic nucleic acid and cell surface (Behr, 1993).

Spermine (Wilson and Bloomfield, 1979) and lipopolyamines (Behr, 1986) condense DNA into submicroscopic particles around charge neutralization. However, these particles do not transfect cells. Only when a large excess of lipopolyamine is present does the process become efficient (Fig. 2). The requirement for "cationic DNA" suggests that interaction with the cell membrane and internalization take place through interaction of the cationic nucleolipidic coat with anionic residues on the cell surface. Indeed, natural lipids are zwitterionic or anionic (never cationic), and due to lipid lateral diffusion in the plane of the membrane, a zipper-like mechanism analogous to the receptor-mediated endocytosis of enveloped viruses could take place (Haywood, 1975), except that here infection would be driven by nonspecific ionic interaction (see scheme, Fig. 8; similar uranyl-mediated DNA translocation into liposomes has been demonstrated: Budker *et al.*, 1987).

### *Dilution of the reporter plasmid into carrier DNA drastically increases the sensitivity of transfection*

For most cationic methods, the DNA–dose/transfection level has been studied by adding variable amounts of DNA to a fixed quantity of cationic vector. Taking into account the absolute requirement for a positively charged complex, such an experimental set-up should give a bell-shaped response where the expected transfection increase with DNA amount will be opposed by the consequences of a decrease in net positive charge; this is indeed observed (Felgner *et al.*, 1987; Gao and Huang, 1991; Zhou *et al.*, 1991). To avoid such interference, we have studied the transfection dose–response with respect to the amount of complex, at a fixed charge ratio, and found a threshold in the microgram range of DNA (Behr *et al.*, 1989). Here the same phenomenon is found (Fig. 3) with a steep increase around 1 μg and a saturation plateau already above ~2 μg. Several hypotheses may explain such a behavior.

One possibility would be that the silent (<1 μg) region was due to a kind of nucleic acid "trap" of microgram size between the cell exterior and the nucleus (for instance a nuclease pool). Thus, we repeated the dose–response experiment by progressive dilution of the plasmid of interest

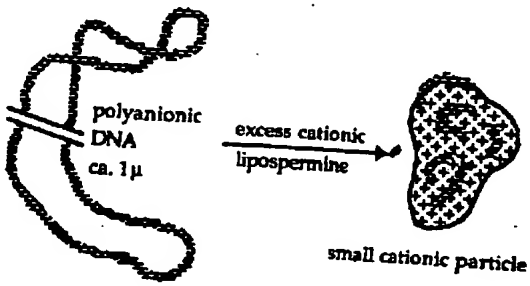


FIG. 7. Schematic view of plasmid DNA compaction by excess lipospermine.

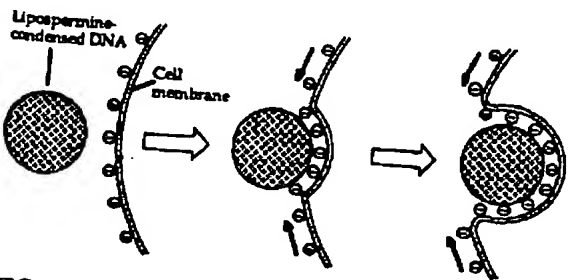


FIG. 8. Schematic representation of the spontaneous endocytosis of a rigid cationic particle.

into carrier DNA to overfill the trap with a constant excess of nucleic acid (5 μg). Under these conditions, the response became linear at low levels (inset in Fig. 3) and reached the same saturation plateau as before, in agreement with our empirical view.

### *Implications and cautionary notes about physiological studies with gene transfer*

From a practical standpoint, plasmid dilution allows extension of the DNA working range far beyond the usual limits, and increases the sensitivity by about two orders of magnitude. Furthermore, because the working DNA range is narrow, caution should be taken in gene expression studies using reporter gene transfection techniques. This is of particular importance when studying transcriptional regulation. Indeed, transfection of a high copy number of reporter gene may then titrate *trans*-acting factors that are rate limiting, and results obtained under such experimental conditions are unlikely to reflect physiological processes.

### *Optimal lipospermine-induced plasmid condensation is highly medium dependent*

Unless there is an optimal size for cell membrane crossing, the smallest particles should transfect the best, so both partners were diluted before encounter, to avoid reticulation (or worse, precipitation). Fast dilution and mixing gives the least good transfection figure, highlighting a kind of necessary "maturation" of the complex (see Fig. 5).

On the other hand, nucleic acid complexing and compaction by polyamines is well-known to be disturbed by other cations (Wilson and Bloomfield, 1979), alkaline-earth cations being more effective than monovalent species (polyanions are expected to interfere too). Hence the observed dependency of transfection on the complex-formation medium: DMEM (and other culture media containing calcium, magnesium or phosphate ions), and serum, of which heparin or albumin (Houen, 1990) may bind lipophilic polycations, should be avoided. The most convenient medium is isotonic with cells and devoid of polyvalent ions: condensation in 150 m*M* NaCl is about 100 times better than in DMEM.

### *An efficient transfection procedure*

In the light of the findings mentioned above, the following recipe should apply to most eukaryotic cells in culture: add 0.1–1 μg of the plasmid of interest to a few micrograms of carrier DNA in 0.1 ml of 150 m*M* NaCl; separately dilute the appropriate amount of lipospermine solution in another 0.1 ml of 150 m*M* NaCl so as to end up with about five times excess cationic charges. After a few minutes, vortex the solutions together and wait for a few minutes before pouring the mixture over ~5 × 10⁵ cells freshly washed and kept in 1 ml of culture medium. Mix gently and after 8–10 hr replace the transfection medium by the appropriate complete culture medium.

Many eukaryotic cell types have been transfected successfully *in vitro* and

thel *et al.*, 1992; Loeffler and Behr, 1993), all wing some general statements to be added: the lipospermine coating method is simple, reproducible, and (when compared) more efficient than most other cation-mediated methods; cytotoxicity and interference with regulatory pathways are low, possibly due to biodegradation. Other nucleic acid binding lipids are being synthesized to circumvent the structural requirements for transfection and hopefully find even more efficient molecules.

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Address reprint requests to:
*Dr. Jean-Paul Behr*
*Laboratoire de Chimie génétique*
*URA 1386 du CNRS*
*Faculté de Pharmacie, B.P.24*
*67401 Strasbourg, France*

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